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Original Article

Microbiology Section

Role of PCR in the Diagnosis of Pulmonary and Extra-Pulmonary Tuberculosis

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ABSTRACT

Introduction: Accurate and early diagnosis is needed for effective management of tuberculosis which remains to be one of the serious public health issues.

Aim: To compare the IS6110 gene based Polymerase Chain Reaction (PCR) with conventional culture on Lowenstein Jensen media for the diagnosis of pulmonary and extra-pulmonary tuberculosis (EPTB).

Materials and Methods: In a prospective study pulmonary and extra-pulmonary specimens from 100 patients clinically suspected with tuberculosis were processed by smear microscopy, culture and IS6110 based PCR following standard procedures. Among the cultured *M. tuberculosis* isolates, drug resistance to isoniazid and rifampicin was studied by line probe assay.

Results: The positivity rates by smear microscopy, culture and PCR were 28(56%), 36(72%) and 37(74%) respectively for suspected pulmonary tuberculosis (PTB) (n=50) and 18(36%), 29(58%), 39(78%) for EPTB (n=50). Sensitivity and specificity of PCR for extra-pulmonary samples in comparison with culture as gold standard was 82.2% and 28.6% respectively but it was 92.1% and 82.2% respectively when clinical diagnosis was considered as gold standard. PCR helped to diagnose 10 extra cases (39 Vs 29) of EPTB when compared to culture. Resistance to isoniazid, rifampicin and multi-drug resistance was observed in 38.6%, 22.8% and 21% respectively. Turn-around-time for PCR was 24-48 hours when compared to culture positivity which is 4-6 weeks.

Conclusion: PCR should be included especially in case of EPTB for timely diagnosis of cases of tuberculosis so that anti-tubercular treatment can be started in time to lower the morbidity of the disease.

Keywords: Anti-mycobacterial drug resistance, Molecular diagnosis, Public health disease

INTRODUCTION

Tuberculosis (TB), an infectious disease caused by the bacillus *Mycobacterium tuberculosis* remains one of the world's deadliest communicable diseases with an estimated global incidence of 9.0 million and 1.5 million deaths in 2013. TB typically affects the lungs but can affect other sites as well referred to as extra-pulmonary TB (EPTB) [1].The commonest presentation of EPTB is the lymph nodes enlargement followed by pleural effusion. Though in immunocompetent patients the involvement of EPTB is seen in 15-20% cases, but in HIV positive individuals more than 50% can be affected [2].

The laboratory diagnosis of tuberculosis still in many parts of world is made by conventional procedures like microscopy and culture but many molecular methods are finding their place recently [3]. Various molecular tools and methods are used for the confirmation of identity of isolates, direct detection of gene sequences from the clinical specimens and also for molecular detection of drug resistance [4]. Molecular methods in comparison to conventional methods provide early, reliable and reproducible results. Moreover, these methods have been used to look for the presence of *M. tuberculosis* directly in clinical samples and have demonstrated reliability and high sensitivity [3]. It has also helped to reduce the delay in diagnostics. PCR for IS6110 has been proved to be a good target considering the presence of multiple copies of this insertion sequence (1-20) in most strains of *M. tuberculosis* complex [5]. Laboratory confirmation of TB and its drug resistance is key to ensure that individuals with signs and symptoms of TB are correctly diagnosed and treated [1]. Timely treatment of tuberculosis patients not only improves patient outcomes, but also helps to decrease the transmission of this infectious disease and more effective public health interventions [6].

A prospective study was conducted to compare the IS6110 gene based PCR with conventional culture on LJ (Lowenstein Jensen) media for the diagnosis of pulmonary and extra-pulmonary tuberculosis. Anti-tubercular drug resistance to isoniazid and rifampicin was assessed by line probe assay.

MATERIALS AND METHODS

A prospective study was conducted from August 2011 to July 2013 in a tertiary care hospital of Coastal Karnataka, India. The study was approved by the Institutional Ethics Committee. One hundred patients clinically suspected with either

pulmonary TB (50) or EPTB (50) were included in the study. Informed consent was obtained at patient enrolment. Patients presenting with cough of more than two weeks duration and chest X-Ray finding suggestive of PTB and patients clinically suspected with EPTB were included in the study. Patients who were previously diagnosed with TB and patients receiving anti-tubercular treatment (ATT) were excluded from the study. Sputum or broncho-alveolar lavage (BAL) fluid samples were processed for PTB. Two sputum samples (one early morning and one spot sample) were collected from each patient suspected of PTB. Lymph node biopsy, bone tissue, pleural fluid, CSF, gastric lavage, urine, bone marrow biopsy and endometrial tissue were processed for EPTB. The tissue samples were put in sterile normal saline and transported to lab. Three consecutive days early morning urine samples were collected for patients suspected of genito-urinary tuberculosis. All the samples received were processed following standard guidelines. Sputum samples were processed by modified Petroff's method. Body fluids were centrifuged at 3000 rpm for 30 minutes and the sediment was used for further testing. Tissue samples were grounded in normal saline and then proceeded further. The processed samples were subjected to microscopy, culture on LJ medium and PCR for detection of *M. tuberculosis*. For microscopy, smears were stained with auramine O and observed under fluorescent microscope. LJ media were used for culture of *M. tuberculosis* and the inoculated media were observed weekly for characteristic growth for eight weeks. PCR was performed for amplification and detection of IS6110 gene in specimens using commercial kits (Genei, Bengaluru, India). *M. tuberculosis* isolates grown in culture from specimens positive by PCR were tested for isoniazid and rifampicin resistance using the line probe assay, Genotype® MTBDR plus (BioMérieux, Marcy I'Etoile, France) as per the manufacturer's protocols for detection of mutations conferring drug resistance.

RESULTS

Sixty-six of the total 100 study subjects were male and the rest 34 were female with M:F::1.9:1. Median age of study group was 39 years. Majority of the patients (58%) were between 18-45 years. The clinical characteristics of the study group are presented in [Table/Fig-1]. Among the patients presenting with signs of PTB, sputum (33, 66%) and BAL fluid (17, 34%) specimens were analysed. Extra-pulmonary specimens processed included 32 (64%) fluids and 18 (36%) tissues from 50 patients. The fluids comprise pleural fluid (12), aspirated pus (8), CSF (7), Gastric lavage (3) and urine (2); whereas the tissues comprise of bone marrow biopsy (8), soft tissue (6) and endometrial tissues (4).

The positivity rates by smear microscopy, culture and PCR were 28(56%), 36(72%) and 37(74%) respectively for suspected PTB (n,50) and 18(36%), 29(58%), 39(78%) for EPTB (n,50). Sensitivity of PCR was better than smear examination in both pulmonary (91.7% Vs 75%) and extra-pulmonary samples (82.2% Vs 51.7%), when culture was considered as gold standard [Table/Fig-2]. But at the same time specificity was observed less for PCR than smear examination. Comparing PCR results with that of clinical diagnosis in case of EPTB as gold standard fetched better sensitivity (92.1%) and specificity (66.7%) [Table/Fig-3].

Characteristic	Number (n)	Percentage
Pulmonary TB group (n=50)		
Cough with expectoration	44	88
Fever	42	84
Chest pain	13	26
Breathlessness	13	26
Hemoptysis	6	12
Co-morbidities		
Diabetes mellitus	17	34
Pneumonia	13	26
Chronic obstructive pulmonary disease	04	08
Smoking	16	32
Alcohol consumption	13	26
Extra Pulmonary TB group (n=50)		

[Table/Fig-1]: Clinical characteristics of the study group

	Pulmonary Samples (50)				Extra-pulmonary samples (50)			
	Smear		PCR		Smear		PCR	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Culture								
Positive	27	9	33	3	15	14	24	5
Negative	1	13	4	10	3	18	15	6
Total	28	22	37	13	18	32	39	11
Sensitivity	75% (57.79% – 87.89%)		91.7% (74.5% - 96.9%)		51.7% (32.5% – 70.54%)		82.2% (64.2% - 94.1%)	
Specificity	92.9% (66% - 98.8%)		71.4% (46.2% - 94.6%)		85.7% (63.6% - 96.7%)		28.6% (11.35% - 52.17%)	
Kappa agreement	0.58		0.65		0.35		0.12	

[Table/Fig-2]: Results of Smear and PCR compared to Culture (gold standard) for Pulmonary and Extra-pulmonary TB (100)

Clinical Diagnosis		PCR		Total
		Positive	Negative	
	Positive	35	3	38
	Negative	4	8	12
Total (n)		39	11	50
Sensitivity of PCR	92.1%			
Specificity of PCR	66.7%			
Kappa agreement	0.61			

[Table/Fig-3]: Showing comparison of PCR and clinical diagnosis (gold standard) in Extra-pulmonary specimens

Anti-mycobacterial drug sensitivity testing of *M.tuberculosis* isolates (57) against isoniazid and rifampicin was performed for 33 pulmonary isolates and 24 extra-pulmonary isolates that were both culture and PCR positive. Isoniazid and rifampicin mono-resistance were observed in 22 (38.6%) and 13 (22.8%) isolates respectively. Multi-drug resistance (MDR) in the study group was observed for 12/57(21%) strains. It was marginally higher in pulmonary group 7/33 (21.2%) as compared to extra-pulmonary group 5/24 (20.8%).

DISCUSSION

The present study showed 58% of TB cases were included in the age group 18-45 years, and male predilection of 66% which is in concordance with the study performed by Zakham et al., [7] which showed 65.9% of total TB cases between 15-45 years of age, with a significant male predilection (59.3%). The relative higher incidence in males can be attributed to more exposure of the male folk to the external environment for their jobs or other related activities when compared to the females or lower notification of cases in female population.But it does not necessarily means that females are more resistant to tuberculosis. It may be due to under-diagnosis or under-reporting, as a result of various social and/or cultural factors often seen in developing countries [8].

The present study has highlighted the presence of diabetes and smoking in one-third of cases. Previous studies have also shown smoking as an important risk factor and that there is strong dose-response relationship between smoking and TB. Nicotine present in cigarettes causes impaired clearance of mucosal secretions, reduced phagocytic ability of alveolar macrophages, and decrease in the immune response and/or reduced CD4+ lymphocytes [9]. Soham Gupta et al., [10] in their study found 30.9% of patients with TB had Diabetes mellitus (DM) followed by other risk factors including smoking (16.9%) and alcoholism (12.6%). A systematic review by Lonnroth K et al., [11], showed the relative risk of 2.94 in pooled studies for the development of tuberculosis where the subjects reported to consume more than 40ml of alcohol per day. They have suggested that direct toxic effects of alcohol on the immune system, micro- and macronutrient deficiency and other alcohol-related medical conditions such as malignancies and depression might lead to development of disease in infected patients [11]. Jethani et al., [12] on the contrary reported 57% tobacco smoking and 35% of alcoholism in their study population.

In developing countries, sputum smear microscopy has been the primary mode of diagnosing pulmonary tuberculosis. In this study sensitivity and specificity of smear microscopy was 75% and 93% for pulmonary isolates which is in concordance with other Asian studies [13]. Sputum smear microscopy is less sensitive when the bacterial load is less than 10,000 organisms/ml of sample and also in extra-pulmonary tuberculosis, paediatric tuberculosis and in HIV/TB coinfections. In our study, sensitivity and specificity of smear microscopy was 52% and 86% for extra-pulmonary isolates.

Even though culture is considered the gold standard in TB diagnostics, growth on solid culture media requires four to six weeks. This delay would negatively affect patient care. To overcome this problem nucleic acid amplification (NAA) techniques are being opted for. A study done by Kibiki et al., [14], the sensitivity and specificity of PCR were 85.7% and 90.9% respectively in pulmonary specimens. Sensitivity and specificity of PCR in our study taking culture as gold standard is 92% and 71% respectively for pulmonary samples with kappa showing good agreement (0.65).

As pointed out by studies globally and in India by (Sekar B et al.,), PCR was more sensitive than conventional culture methods for EPTB [15]. Confirmation of EPTB is challenging for a number of reasons: the difficulty to obtain an adequate sample; the apportioning of the sample for various diagnostic tests resulting in non-uniform distribution of microorganisms; the pauci-bacillary nature of the specimens; and the lack of an efficient sample processing technique universally applicable on all types of extra-pulmonary samples. All these limitations cause poor contribution of conventional bacteriological techniques in the establishment of diagnosis of EPTB [16]. Diagnoses of EPTB without microbiological confirmation may result in over-diagnosis. This has stimulated the application of polymerase chain reaction in the laboratory diagnosis of EPTB. In the present study, the sensitivity and specificity of 82.2% and 28.6% respectively was observed for PCR for all extra-pulmonary specimens with culture as gold standard against the results of 92.1% and 66.7% considering clinical diagnosis as gold standard. Statistically kappa showed good agreement of 0.61 in latter case. Due to low yield of positive culture in paucibacillary EPTB cases, many researchers have used clinical diagnosis as gold standard earlier too [14].

In the present study, 19 cases (4 pulmonary and 15 extra-pulmonary samples) have positive PCR but negative culture results. The negative culture findings can be due to low bacillary load in samples or due to harsh decontamination procedures. However negative culture cannot preclude a clinical diagnosis of tuberculosis particularly in extra-pulmonary samples. Out of these 19 cases, 14 were clinically suggestive of TB and hence treated with ATT. At the same time, in the present study four samples (2 CSF, 1 pleural fluid and 1 urine sample) of 50 extra-pulmonary TB samples were culture positive but PCR negative. Out of these one was smear positive (2+), two were

retro-positive and one was not clinically treated for TB. The negative results in PCR may be due to presence of inhibitory substances present in clinical specimens, or absence of IS6110 gene in these strains.

Results of drug susceptibility testing revealed the resistance to isoniazid was higher (38.6%) as compared to rifampicin (22.8%). Earlier researchers have also reported the higher resistance in isoniazid as compare to rifampicin [16]. Multi-drug resistance was observed in the present study as 21.2% and 20.8% in PTB and EPTB cases respectively. A study done from a south Indian tertiary hospital [17] has revealed the MDR cases 44% and 20.4% in PTB and EPTB respectively. Variation in results can be explained by the existing global differences in drug resistance pattern.

The limitation of the study is the small sample size for both pulmonary and extrapulmonary samples. The follow up of the cases was not done in the study.

CONCLUSION

The present study validates the usefulness of PCR in the diagnosis of tuberculosis especially in paucibacillary EPTB cases where conventional methods may miss the diagnosis. Though PCR helps in early diagnosis of disease, the results of the PCR should be correlated with other diagnostic modalities to substantiate the diagnosis. The emerging higher resistance as observed in this study highlights the need for anti mycobacterial drug sensitivity testing before initiation of ATT.

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